Kluyveromyces lactis Host-vector System

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Introduction

The present application seeks to qualify the yeast *Kluyveromyces lactis* and associated vectors as an NIH-approved host-vector system. *K. lactis* has a 20+ year history of safe use as an expression system in regulated food industry processes and has become an important genetic model organism in fungal genetics akin to *S. cerevisiae* and *Schizosaccharomyces pombe*. Here we summarize what is known about the history, and use of *K. lactis* in the food industry, its taxonomy, strain origin and vector design. We also refer the reader to two extensive review articles that are cited and summarized in this document and are included with our application (see ref. 1, 2).

K. lactis in Biotechnology and the Food Industry

K. lactis and products derived from *K. lactis* have been safely used in food industry applications for almost 50 years (extensively reviewed in ref. 1). In notable examples, dried *K. lactis* powder was used as a dietary protein supplement in the 1960's and 1970's, a purified native lactase isolated from *K. lactis* has been (and still is) used to orally treat lactose intolerance since the 1980's, and recombinant bovine chymosin is produced from engineered *K. lactis* for use in cheese manufacturing.

In the early 1980's, *K. lactis* became one of the first yeasts to be transformed by foreign DNA in the laboratory (3). This led to the development of a host-vector expression system by the Dutch food company Gist-brocades BV (now DSM Food Specialties) that culminated with the production of the important food industry enzyme, recombinant chymosin (a cheese rennant). A large industrial fermentation and manufacturing process was established for production of this enzyme (4) and in 1988, *K. lactis*-produced chymosin became the first recombinant protein approved for use in food applications. This *K. lactis* produced enzyme was also granted GRAS status by the FDA (5).

In the early 1990's, other industrial yeast expression systems including *Pichia* and *Hansenula* made a transition from big industry to life sciences research. However, due to the lucrative nature of the patents covering the use of the *K. lactis* host-vector system (for production of prochymosin), it remained exclusively a food industry technology. Over the years, several academic labs in the US and Europe have used *K. lactis* for the expression of various recombinant proteins. As of 2006, over 45 examples of heterologous protein expression using *K. lactis* have been published (references are summarized in Table I of ref. 2). Advantages of *K. lactis* as an expression system are that it grows to extremely high density in bioreactors, it does not require methanol for high gene expression (as with *Pichia pastoris*), it supports protein expression from both stable episomal vectors and integrative vectors, and it can be easily manipulated with genetic techniques and media originally devised for manipulation and growth of *S. cerevisiae*.

K. lactis taxonomy, morphology and physiology

K. lactis is a "budding yeast" from the phylum Ascomycota. It is evolutionarily closely related to the well-known baker's yeast *S. cerevisiae*. In fact, *K. lactis* was originally

classified as *Saccharomyces lactis*, but this taxonomic classification was changed in 1984 to *Kluyveromyces marxianus* var. *lactis,* with *K. lactis* being the accepted shortened notation (6). Both organisms share many similarities and several key differences.

Both organisms are morphologically similar with *K. lactis* cells generally being smaller than *S. cerevisiae* cells. Both organisms divide by budding and can exist in either in a haploid or diploid state. Diploids of either organism can undergo sexual reproduction and generate four haploid ascospores, the products of meiotic segregation. Similar to *S. cerevisiae*, *K. lactis* haploids can have either **a** or α mating types. However, *K. lactis* haploids cannot mate with *S. cerevisiae* haploids. No other modes of genetic transmission for *K. lactis* have been described.

In nature, *S. cerevisae* and *K. lactis* have different ecological niches that primarily reflect physiological differences in how the two organisms metabolize sugars. *S. cerevisiae* thrives in rotting fruit where glucose concentration is high. It can also produce ethanol aerobically as a metabolic byproduct when glucose concentration is high, a process known as the “Crabtree effect”. In contrast, *K. lactis* possesses a highly expressed lactase that degrades milk sugar (lactose) to galactose and glucose. This enzyme is absent in *S. cerevisiae*. Thus, *K. lactis* is a natural and indispensable component of cultured dairy processes where lactose concentration is very high. Wild *K. lactis* isolates have exclusively been isolated from diary processes such as yogurt, cheese and buttermilk making. Additionally, *K. lactis* is Crabtree-negative and does not produce ethanol aerobically.

Strains and Vectors

The academic community settled on the prototrophic strain CBS2359 (ATCC 8585) as the type strain for the *K. lactis* genome sequencing project. GG799 is a wild-type haploid strain originally isolated by Gist-brocades BV from a dairy process. At New England Biolabs, over a dozen different GG799 genes have been cloned and sequenced with little or no sequence variation from corresponding gene sequences reported for CBS2359. GG799 has been shown to have excellent ability to produce heterologous proteins and propagate to very high cell density in bioreactors, making it an attractive “industrial” background. GG799 cells have no auxotrophic markers or other genetic modifications (Colussi and Taron, 2005). Strains carrying common auxotrophies (e.g. uracil auxotrophy by deletion of *URA3*), protease gene deletions (7) or glycosylation gene deletion have been constructed in the GG799 background.

Three vector systems have been described for *K. lactis* (reviewed in ref. 2). Early more primitive vectors were derived from native linear *K. lactis* plasmids pKL1 and pKL2. They were unstable and are seldom used today. Episomal vectors (e.g. pKD1-based vectors) are commonly used in *K. lactis* (reviewed in ref. 2). These plasmids are analogous to *S. cerevisiae* 2-micron vectors and are stably maintained in *K. lactis*. Finally, as with other yeast and filamentous fungal host-vector systems, vectors that stably integrate at a site-specific locus in the host chromosome are also used. In the latter case, NEB has designed vectors (pKLAC1, pKLAC2, pKLMF-EK, pKLMF-FX, pKLCF-n, pKLCF-c) that stably and efficiently integrate into the *LAC4* (lactase) region of the *K. lactis* chromosome (8,9). Sequences for these vectors have the following GenBank accession numbers, respectively: AY968582, EU196354, FJ010196, FJ010197, HQ214066, HQ236722. Each of these vectors derives from pGBN1, an

integrative expression vector developed at DSM Food Specialties (5). Selections for maintenance of all *K. lactis* vectors are akin to those routinely used in *S. cerevisiae* or other fungi and include complementation of nutritional auxotrophies (e.g. *ura3*, *his3* etc), the use of antibiotic resistance genes (e.g. kanamycin, neomycin) or nitrogen source selection (e.g. growth on acetamide).

Strains are propagated in both shake flasks and bioreactors using media and methods nearly identical to those of *S. cerevisiae*. Introduction of foreign DNA into *K. lactis* backgrounds is performed by electroporation or by chemical (lithium acetate/polyethylene glycol) transformation using protocols developed for *S. cerevisiae* manipulation.

References

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